

Figure 3. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for decane, undecane, dodecane and methyl decanoate. The upper right corner of each graph shows the structure of the analyte.

produced $\delta^{13}\text{C}$ values that were signal dependent. Our initial investigations showed that although the $\delta^{13}\text{C}$ values produced for the VG mix were approximately non-signal dependent to $\pm 0.3\text{‰}$ for the ion beam intensities between 1×10^{-9} and 1×10^{-8} A, the sample accuracy and precision soon degraded significantly between 1×10^{-10} and 1×10^{-9} A. A series of injections was undertaken to produce I^{44} intensities of between 1×10^{-10} and 8×10^{-8} A. Figure 3 illustrates that the $\delta^{13}\text{C}$ values produced for decane, undecane, dodecane and methyl decanoate are exponentially dependent on the I^{44} intensity. The delta value (Δ) plotted represents the difference between the theoretical value given by Micromass UK Ltd. and the values recorded by the IRMS. All four compounds showed a positive Δ of between 1.6 and 2‰ when the ion beam signal was smaller than 5×10^{-10} A. This enrichment decreased with increasing I^{44} intensity. Figure 3 demonstrates that the theoretical $\delta^{13}\text{C}$ value is approached when the I^{44} intensity is between 5.5×10^{-9} and 7×10^{-9} A. This is because the $\delta^{13}\text{C}$ value of the reference gas used in the IRMS was cross-calibrated using VG mix at I^{44} intensities of 6×10^{-9} to 6.5×10^{-9} A. The relative molecular masses of these compounds varied from 142 to 172 Da. The logarithmic regression observed in Fig. 3 does not appear to be mass dependent. The MSD successfully identified all compounds plotted in Fig. 3 to at least a 93% confidence level.

Bioremediation of phenol is currently ongoing within the EERC.¹⁸ Phenol was therefore selected to study the effect of signal intensity on the $\delta^{13}\text{C}$ value recorded for a smaller molecule. Sample sizes analysed were again varied to produce I^{44} intensities of 1×10^{-10} – 1×10^{-8} A. Figure 4(a) illustrates that the $\delta^{13}\text{C}$ values produced were again

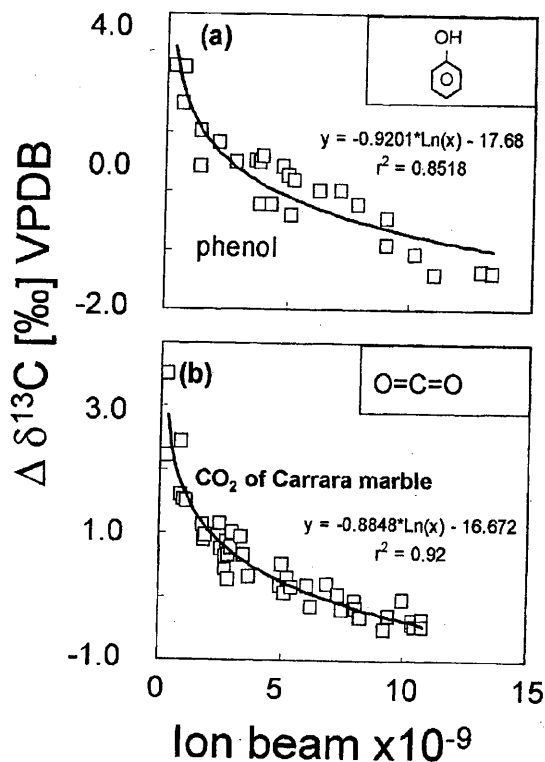


Figure 4. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for phenol and CO_2 from Carrara marble. The upper right corner of each graph shows the structure of the analyte.

signal dependent. The $\delta^{13}\text{C}$ value becomes more enriched with decreasing sample size, reaching a maximum enrichment of 1.2‰ at an I^{44} intensity of 7×10^{-10} A. The theoretical $\delta^{13}\text{C}$ value of phenol (-28.5‰) is approached when the I^{44} intensity is 6.8×10^{-9} A.

CO₂ standards

Our aim was also to investigate the role of the combustion tube in the signal dependence trend observed. A series of CO₂ analyses derived from the acidification of Carrara marble were performed. Injecting CO₂ directly onto the column with identical operational conditions for the combustion interface obviously negates the necessity of CuO in the combustion interface to oxidise the carbon present in the sample. Figure 4(b) shows the $\delta^{13}\text{C}$ value plotted against ion beam size. The Carrara marble standard shows a maximum enrichment of 2.5‰ for an ion beam size of 3×10^{-10} A. The true value of the standard (2.4‰) is approached for an ion beam size of 6.5×10^{-9} A.

DISCUSSION

This investigation outlines the successful coupling of two independent detectors, an MSD and an IRMS to a GC. When using standard gas chromatography/mass spectrometric analysis it is important to optimise the detector to enable quantification and qualification of the smallest possible sample size. The IRMS used in our system has been optimised to operate at a high sensitivity. This now enables the quantification, qualification and $\delta^{13}\text{C}$ values of compounds to be recorded from one sample analysis.

After compromising the signal independence by tuning the IRMS for higher sensitivity, a logarithmic relationship occurs between the $\delta^{13}\text{C}$ value and the I^{44} intensity for all four compounds in Fig. 3. By manipulating the equation best describing the points ($y = m^* \ln(x) + c$) in Fig. 3, Eqn. 2 is derived:

$$\delta^{13}\text{C}_{\text{true}} = \delta^{13}\text{C}_{\text{actual}} - ((m^* \ln(I^{44})) + c) \quad (2)$$

We were then able to correct the $\delta^{13}\text{C}$ values obtained for any I^{44} between 2×10^{-10} and 7×10^{-9} A. Using this correction equation, the compounds in Fig. 3 produced $\delta^{13}\text{C}$ values to an accuracy of <0.01‰ and a precision of between 0.13 and 0.23‰. Corrected phenol $\delta^{13}\text{C}$ values were also calculated using Eqn. 2. Over two orders of magnitude the accuracy using this technique is <0.01‰ with a precision of 0.3‰. This precision is not particularly good. This could be an artefact of sample degradation over time. The results were obtained over two days and phenol has been shown to degrade as a function of time in ethyl acetate.²⁰

The $\delta^{13}\text{C}$ values of the organic compounds investigated all display a signal dependence relative to I^{44} intensity. A possible source of instrumental error could be the combustion interface.⁸ To elucidate whether this may be the case CO₂ was injected directly onto the column. The Carrara marble data showed the same logarithmic signal dependence shown by compounds that were injected on-column as liquids and combusted on the CuO interface. The $\delta^{13}\text{C}$ values could therefore also be corrected by Eqn. 2. When the correction was performed, the $\delta^{13}\text{C}$ values obtained were accurate to 0.01‰ with a precision of 0.25‰ from 2.2×10^{-10} to 1.1×10^{-8} A. The IRMS does have the potential for very

Table 2. Measured and corrected $\delta^{13}\text{C}$ values of decane at variable I^{44} beam intensities. The average of the latter shows a significant increase in precision and coincides with the theoretical value (high accuracy)

I^{44} beam	$\delta^{13}\text{C}$ measured	$\delta^{13}\text{C}$ corrected
1.19E-10	-26.96	-28.87
1.61E-10	-26.68	-28.45
4.00E-10	-27.18	-28.52
4.76E-10	-27.51	-28.77
4.82E-10	-27.11	-28.36
5.49E-10	-27.36	-28.55
7.47E-10	-27.47	-28.51
8.46E-10	-27.88	-28.86
1.11E-09	-27.78	-28.64
1.17E-09	-27.82	-28.65
1.48E-09	-27.93	-28.65
2.17E-09	-27.97	-28.51
2.18E-09	-28.13	-28.67
2.36E-09	-28.02	-28.52
2.43E-09	-28.09	-28.58
2.53E-09	-28.00	-28.47
2.64E-09	-28.36	-28.81
2.88E-09	-28.14	-28.55
3.27E-09	-28.04	-28.39
3.66E-09	-28.46	-28.75
3.95E-09	-28.34	-28.60
4.01E-09	-28.19	-28.44
4.23E-09	-28.40	-28.62
4.28E-09	-28.46	-28.68
4.52E-09	-28.44	-28.63
4.55E-09	-28.41	-28.60
4.88E-09	-28.40	-28.56
5.70E-09	-28.65	-28.73
6.38E-09	-28.58	-28.61
6.57E-09	-28.73	-28.75
Average	-27.98	-28.61
$1\sigma^*$	0.53	0.13

* Error to one standard deviation.

good internal precision. Table 2 shows the $\delta^{13}\text{C}$ values for analysis of CO₂ from decane over a range of sample sizes. Before adjusting the $\delta^{13}\text{C}$ values for signal size, the internal precision of the instrument is 0.53‰ for a range of I^{44} between 1.2×10^{-10} to 6.6×10^{-9} A. After correction, the average of the calculated value exactly matches the theoretical value of -28.61‰, while the precision decreases to 0.13‰.

As can be seen from the equations in Figs 3 and 4, the trends for the $\delta^{13}\text{C}$ values of the compounds in the VG mix, phenol and CO₂ are similar. Figure 5 illustrates all data points recorded ($\Delta = \delta^{13}\text{C}_{\text{actual}} - \delta^{13}\text{C}_{\text{true}}$) plotted as a function of the I^{44} intensity. The r^2 value in the equation describing the signal dependence is 0.72. This suggests that an overall correction factor could be used for all data to enhance $\delta^{13}\text{C}$ accuracy and precision over the I^{44} ranges of 2×10^{-10} to 1×10^{-8} A. However, we have found from the data presented that compound specific correction achieves better results.

The data presented here clearly show that the combustion interface performance can not account for the trends outlined in this paper. The water trap, if properly maintained, is of a reliable design and is also an unlikely source of any routine error. With decreasing sample size, any air in the system, and in particular nitrogen, will become a larger proportion of the total sample size. The possible contribution of any incompletely oxidised N₂ in the form of

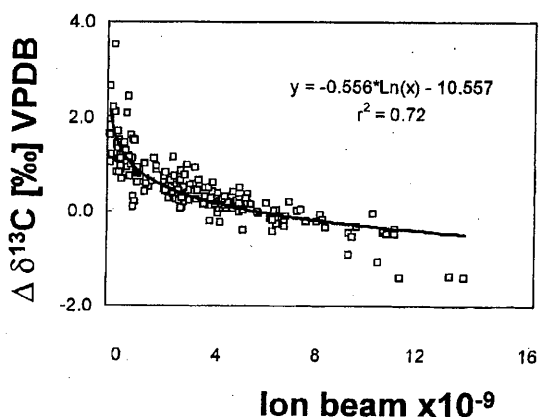


Figure 5. Ion beam strength versus the difference in isotopic composition between measured and theoretical values ($\Delta \delta^{13}\text{C}_{\text{DIC}}$) for decane, undecane, dodecane and methyl decanoate, phenol and CO_2 from Carrara marble plotted in one graph.

N_2O was therefore of concern. However, any addition of ionised N_2O to the I^{44} beam would produce depleted $\delta^{13}\text{C}$ values with decreasing I^{44} intensity, the opposite of the trend observed. It is interesting to note that the application of a logarithmic correction to sample size dependant $\delta^{13}\text{C}$ values has also been reported for dual inlet measurements.²¹ However, the major difference between continuous flow and dual inlet sample introduction makes the previously published explanation inappropriate. One possible explanation for the signal dependence may be mass diffusion in the IRMS source. There is a flow of CO_2 and helium (He) into the ion source. As the ratio of CO_2 to He changes (i.e. decreasing beam I^{44} strength), there may be an increasing potential to accentuate the diffusional fractionation of mass 44, 45 and 46 and therefore cause variation in residence times of these ions within the source. Further work is needed to elucidate the exact nature of the observed phenomenon.

CONCLUSIONS

A GC has been coupled successfully with two detectors, an MSD and an IRMS. The nature of the flow splits in the instrumental coupling make it desirable to achieve a higher degree of sensitivity than may normally be required when using a routine GC/IRMS. The signal dependent trend observed is similar for all the compounds studied in this paper. However, the signal dependence that occurs from a high sensitivity source tuning can be corrected for. The

results indicate that a general correction equation could be used to correct $\delta^{13}\text{C}$ values for I^{44} intensity. However, where possible, compound specific correction provides the best accuracy and precision. This allows routine continuous flow analysis using a GC/MSD/IRMS system to produce very accurate results to a precision approaching 0.1‰ over two orders of I^{44} magnitude.

Acknowledgements

M. Carey, N. Ogle and J. Hamilton are thanked for their technical assistance. This work was supported by grants from the QUESTOR Industrial Board, Queen's University Belfast, and EPSRC grants L85183 and M26374.

REFERENCES

1. W. A. Brandt, *J. Mass Spectrom.* **31**, 225 (1994).
2. C. R. McKinney, J. M. McCrea and S. Epstein, *Rev. Sci. Instrum.* **21**, 724 (1950).
3. G. St-Jean, Introduction to Stable Isotopes, *The 5th Canadian Continuous Flow Isotope Ratio Mass Spectrometry Workshop, Ottawa* (1988).
4. J. A. Hall, R. M. Kalin, M. J. Larkin, C. C. R. Allen and D. B. Harper, *Org. Geochem.* (in press).
5. P. K. Aggerwal, M. E. Fuller, M. E. Gurgas, J. F. Manning and M. A. Dillon, *Env. Sci. Tech.* **31**, 590 (1991).
6. M. Sano, Y. J. Yotsui, H. Abe and S. Sasaki, *Biomed. Mass Spectrom.* **3**, 1 (1976).
7. D. E. Mathews and J. M. Hayes, *Anal. Chem.* **50**, 1465 (1978).
8. P. A. Eakin, A. E. Fallick and J. Gerk, *Chem. Geol.* **101**, 71 (1992).
9. D. A. Merritt, W. A. Brand and J. M. Hayes, *Org. Geochem.* **21**, 573 (1994).
10. W. Meier-Augenstein, *J. High Res.* **18**, 28 (1995).
11. J. E. Landmeyer, D. A. Vroblesky and F. H. Chappelle, *Env. Sci. Tech.* **30**, 1120 (1996).
12. M. Wittaker, S. T. J. Pollard and T. E. Fallick, *Env. Tech.* **16**, 1009 (1995).
13. W. Meier-Augenstein, W. Brand, G. F. Hoffmann and D. Rating, *Biolog. Mass. Spectrom.* **23**, 376 (1994).
14. H. S. Dempster, B. Sherwood Lollar and S. Feenstra, *Env. Sci. Tech.* **31**, 3193 (1997).
15. C. C. R. Allen, D. R. Boyd, M. J. Larkin, K. A. Reid, N. D. Sharma and K. Wilson, *Appl. Env. Microbiol.* **63**, 151 (1997).
16. R. C. Bayley and S. Dagley, *Biochem. J.* **111**, 111 (1969).
17. K. Rozanski, W. Stichler, R. Gonfiantini, E. M. Scott, R. P. Beukens, B. Kromer and J. Van der Plicht, *Radiocarbon* **34**, 506 (1992).
18. R. M. Kalin, J. A. Hall, U. N. Fitzgerald, Environmental Engineering Research Centre, unpublished data (1998).
19. Micromass UK Ltd., Isochrom GC User Manual, issue 3, pp. 28–29 (1996).
20. J. A. Hall, Environmental Engineering Research Centre, unpublished data (1998).
21. C. M. Scrimgeour, K. Smith and M. J. Rennie, *Biomed. Environ. Mass. Spectrom.* **15**, 369 (1988).

Use of gas chromatography–combustion–isotope ratio mass spectrometry in nutrition and metabolic research

Wolfram Meier-Augenstein

Linking gas chromatography via an on-line combustion interface to isotope ratio mass spectrometry has opened the door to high-precision compound-specific isotope analysis. For this reason, gas chromatography–combustion–isotope ratio mass spectrometry is now increasingly employed in metabolic and nutritional research because it offers a reliable and risk-free alternative to the use of radioactive tracers. *Curr Opin Clin Nutr Metab Care* 2:000–000. © 1999 Lippincott Williams & Wilkins.

Correspondence to Wolfram Meier-Augenstein, CChem MRSC, Senior Research Fellow, University of Dundee, Department of Anatomy and Physiology, OMS, Small's Wynd, Dundee, DD1 4HN, UK
Tel: +44 0 1382 345124; fax: +44 0 1382 345514; e-mail: w.meieraugenstein@dundee.ac.uk

Current Opinion in Clinical Nutrition and Metabolic Care 1999, 2:000–000

Abbreviations

APE	atom% excess
BCAA	branched-chain amino acids
GC–C–IRMS	gas chromatography–combustion–isotope ratio mass spectrometry
CSIA	compound-specific isotope analysis
GC–MS	gas chromatography–mass spectrometry
IRMS	isotope ratio mass spectrometry
SIM	selected ion monitoring mode

© 1999 Lippincott Williams & Wilkins
1363–1950

Introduction

One of the reasons for the increasing appeal of gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) is the fact that, as a result of this technology, stable isotope labelled tracers have become a viable alternative to radioactive tracers, a fact that is of particular relevance when dealing with paediatric patients. With the exception of deuterium (^2H) when used in high concentrations, heavier stable isotopes such as ^{13}C , ^{15}N and ^{18}O do not cause any adverse physiological effects, even at high enrichment levels [1]. Isotope ratio mass spectrometry (IRMS) is an analytical mass spectroscopic technology, for it provides quantitative information rather than structural information on a given compound. The application of high-precision compound-specific isotope analysis (CSIA) in nutrition and metabolic research can be roughly divided into two areas. One area of application is concerned with studies to measure the rate of intermediary metabolism, the other aims to trace metabolic pathways using labelled precursor compounds at high enrichment levels to improve detection limits. This article aims to present an overview of the work in these areas during the past 4 years. Because this is the first time that GC–C–IRMS is presented in this forum, some emphasis is also placed on the methodological aspects and constraints of this hyphenated technique.

Practical issues concerning gas chromatography–combustion–isotope ratio mass spectrometry

In contrast to organic mass spectrometers that yield structural information by scanning a mass range over several hundred Daltons for characteristic fragment ions, IRMS instruments achieve a highly accurate and precise measurement of isotopic abundance at the expense of the flexibility of scanning mass spectrometers (Table 1). Because gas chromatography–mass spectrometry (GC–MS) can be used to measure stable isotope enrichment, the question arises as to why one should embrace GC–C–IRMS. Scanning mass spectrometers use a single detector and therefore cannot simultaneously detect particular isotope pairs for isotope ratio measurement. For isotope ratio measurement, the mass spectrometer is best operated in selected ion monitoring mode (SIM) to optimize the sensitivity to selected masses. Even in SIM mode, the limited accuracy and precision of such isotope ratio measurements impose a minimum working enrichment for ^{13}C and ^{15}N of at least 0.5 atom% excess (APE) [2,3]. In other words, organic mass spectrometry cannot

provide reliable quantitative information in cases in which a low turnover or low rate of incorporation results in isotopic enrichment of less than 0.5 APE.

In contrast, GC-C-IRMS can measure isotopic composition at low enrichment and natural abundance levels. This means that minute variations in very small amounts of the heavier isotope are detected in the presence of large amounts of the lighter isotope. Because the small variations of the heavier isotope habitually measured by IRMS are of the order of -0.07 to $+1.09$ APE, the δ -notation in units of per mil (‰) has been adopted to report changes in isotopic abundance as a per mil deviation compared with a designated isotopic standard:

$$\delta_s = [(R_s - R_{std})/R_{std}] \times 1000 \text{ [‰]}$$

where R_s is the measured isotope ratio for the sample and R_{std} is the measured isotope ratio for the standard. To give a convenient rule-of-thumb approximation, in the δ -notation, a ^{13}C enrichment in the range of -0.033 to $+0.0549$ APE corresponds to a $\delta^{13}\text{C}$ value range of -30% to $+50\%$. A change of $+1\%$ is approximately equivalent to a change of 0.001 APE and 0.0003 APE for ^{13}C and ^{15}N , respectively.

The sensitivity of GC-C-IRMS is such that tracer/tracee (mol/mol) ratios down to 10^{-5} can be reliably detected [4]; in the same review, Brenna *et al.* also provide an in-depth discussion of notations and elementary calcula-

tions such as mass balance and pool mixing equations.

As a result of its high sensitivity, GC-C-IRMS depends on careful sample preparation and high-resolution capillary gas chromatography [5**]. Demands on sample size, sample derivatization, quality of gas chromatography separation, interface design and isotopic calibration have been discussed in a number of reviews [2,3,5**,6-8,9**].

High-precision compound-specific isotope analysis of ^{13}C isotopic abundance

The great clinical and scientific potential of ^{13}C tracer techniques for nutritional and metabolic research in paediatric patients has been discussed recently [10**]. Several comparative studies have demonstrated the considerable advantages of GC-C-IRMS in nutritional and metabolic research. Pont *et al.* [11] measured ^{13}C enrichment in the cholesterol of rabbit low-density lipoprotein after the injection of 3 mg of [3,4- ^{13}C]cholesterol. They compared the accuracy and precision, detection limits and dynamic range (ranging from -22 to $+760$) of GC-C-IRMS and GC-MS measurements and found GC-C-IRMS to be more accurate and reproducible, especially at lower enrichment levels. Highly linear calibration curves for ^{13}C enrichment were also reported from a study with branched-chain amino acids (BCAA) [12]. In that study, slopes of 0.98 and 1.04 were observed for enrichment ranges $0-0.14$ APE and $0-$

Table 1. Typical features and specifications of mass spectrometry systems used for stable isotope analysis

	GC-MS	Elemental analyser-IRMS	GC-C-IRMS
Sample introduction	Injection of liquid (or gaseous) sample matrix on to GC column	Solid (and dry) sample in tin capsules	Injection of liquid (or gaseous) sample matrix on to GC column
Sample separation	Yes, by gas chromatography	No	Yes, by gas chromatography
Sample manipulation before mass spectroscopic analysis	None	Combustion/reduction of sample into CO_2 and N_2 in the elemental analyser	Combustion/reduction of compounds into CO_2 and N_2 in the interface
Interface	Heated transfer capillary directly connected to ion source	Transfer capillary with open split	Capillary, incorporating wide bore combustion/reduction furnaces
Mass analysers	Quadrupole	Magnet	Magnet
Detector	One electron multiplier	Triple Faraday cup collector	Triple Faraday cup collector
Mode of charged mass	SIM, switching between e.g. M^+ and $[\text{M}+1]^+$ ($[\text{M}+1]^+/ \text{M}^+$ ratios are calculated on the basis of measured ion current)	Simultaneous detection of particles with three adjacent masses, e.g. m/z 44, 45 and 46 for $^{12}\text{C}^{16}\text{O}_2$, $^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{18}\text{O}^{16}\text{O}$, respectively	Simultaneous detection of particles with three adjacent masses, e.g. m/z 28, 29 and 30 for $^{14}\text{N}^{14}\text{N}$, $^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$, respectively
Detection		-0.1 – $+2.0$	-0.1 – $+2.0$
Measureable enrichment range in [APE]	$+0.5$ – 100^a		
Sample size requirement	≤ 1 pmol	0.1 – 5 μmol^b	0.1 – 5 nmol ^c
CSIA	Yes, with a precision of 0.05 atom%	No ^d	Yes, with a precision of 0.0002 atom%

^aUsing a multiply labelled tracer (e.g. [$^2\text{H}_5$]phenylalanine) and measuring $[\text{M}+n]^+/ \text{M}^+$ ratios (e.g. $[\text{M}+5]^+/ \text{M}^+$), enrichments down to 0.2 APE can be reliably detected.

^bThe required sample size depends on the type of isotope analysis. Analysis of ^{13}C enrichment requires only small amounts of material because of the high abundance of carbon in organic compounds. The opposite is true for the analysis of ^{15}N enrichment, because of the low abundance of nitrogen in organic compounds. Furthermore, for compounds containing only one atom of nitrogen, two moleequivalents of compound have to be combusted to generate one moleequivalent of N_2 .

^cIn addition to the considerations mentioned above, it should be noted that compound-specific isotope analysis (CSIA) by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) requires the injection of 0.1–5 nmol per individual compound to be analysed.

^dUsing elemental analyser-IRMS, CSIA is only possible for off-line isolated and purified compounds. Here, the precision is 0.001 atom%.

APE, Atom% excess; GC-MS, gas chromatography-mass spectrometry; SIM, selected ion monitoring mode.

8 APE, respectively. Parker *et al.* [13] showed that GC-C-IRMS permits the use of low doses of β -[U- ^{13}C]carotene (≤ 2 mg), which do not perturb endogenous pool sizes of β -carotene or retinol. Employing ^{13}C tracers at low levels of enrichment, reliable data were obtained in studies of the kinetics of glycoprotein neutral sugars [14] and urea [15]. Bunt *et al.* [16*] used [U- ^{13}C]glucose as a precursor to obtain data on endogenous surfactant production and turnover by measuring ^{13}C -enrichment of palmitic acid in phosphatidylcholine palmitate. GC-C-IRMS was also used to determine the range of natural isotope abundance of ^{13}C in six serum fatty acids from humans on a controlled diet [17].

Compound-specific isotope analysis of ^{13}C labelled fatty acids

The high sensitivity of GC-C-IRMS has been increasingly exploited in metabolic studies investigating the turnover, incorporation and synthesis processes of fatty acids *in vivo*, which could only previously be investigated either with stable isotope tracers at high enrichment levels using GC-MS, or not at all [18].

GC-C-IRMS, capable of measuring naturally occurring differences in isotope ratios, determined the extent to which neonates and very low birthweight premature infants could synthesize arachidonic acid, which is essential for their growing tissues, from dietary fatty acids. Demmelmair *et al.* [19] showed that in neonates fed on a phenylalanine-free diet, on average 23% of free plasma arachidonic acid on study day 4 originated from infantile linoleic acid conversion. Carnielli *et al.* [20] added linoleic acid and linolenic acid, both ^{13}C -labelled, to the formula diet that was administered continuously for 48 h (birthweight 1.17 ± 0.12 kg; gestational age 28.4 ± 1.3 weeks). The authors demonstrated that both tracers were rapidly incorporated into plasma phospholipids and that their metabolic products including arachidonic acid and docosahexaenoic acid became highly enriched with ^{13}C . The incorporation of ^{13}C -octanoic acid into plasma triglycerides (10% of the enrichment of the diet), noticeably into myristic and palmitic acid, by very low-weight preterm infants was reported earlier by the same group [21].

The majority of ^{13}C tracer studies published have dealt with various aspects of fatty acid biochemistry. The bioequivalence of dietary α -linolenic and docosahexaenoic acid as substrates for brain and retinal n3 fatty acid accretion during the brain growth spurt [22,23*], metabolism of triglycerides [24], transport and turnover of free saturated [25,26,27*] and unsaturated fatty acids [28-30,31*]. While studying the metabolism of ^{13}C -labelled polyunsaturated fatty acids by ^{13}C -nuclear magnetic resonance, using GC-C-IRMS, Cunnane *et al.* [32] found low levels of ^{13}C -labelled γ -linolenic acid

in the brain phospholipids of suckling rat pups that could not be detected by ^{13}C -nuclear magnetic resonance.

Unlike GC-MS, in which increasing the amount of label has no general effect on detection limits, in GC-C-IRMS, increasing label enrichment in precursor compounds produces significantly improved detection limits. Taking advantage of this increased traceability, Rhee *et al.* [33] quantified the desaturation of [U- ^{13}C]18:0 and [U- ^{13}C]16:0 in whole plasma and lipoprotein of adults after the administration of 30 mg oral doses (< 0.5 mg/kg). Su and Brenna [34*] reported the simultaneous measurement of desaturase activities by monitoring the increase in product from the reactions [U- ^{13}C]18:2n6 \rightarrow [U- ^{13}C]18:3n6 and [U- ^{13}C]16:0 \rightarrow [U- ^{13}C]16:1n7, respectively.

Using [U- ^{13}C] α -linolenic acid, Sheaff *et al.* [35] were able to demonstrate that high dietary levels of linoleic acid did not depress the conversion of α -linolenate into docosahexaenoate. Three years later, Menard *et al.* [36*] showed that [U- ^{13}C] α -linolenic acid is not solely a source of docosahexaenoic acid. They found that, owing to a high rate of β -oxidation and carbon recycling, [U- ^{13}C] α -linolenate was utilized in the de-novo synthesis of cholesterol and palmitate.

Compound-specific isotope analysis of ^{13}C -labelled amino acids

In spite of the wide usage that GC-C-IRMS enjoys in research concerned with fatty acid and surfactant metabolism, most research groups interested in amino acid metabolism and protein turnover still seem to favour traditional GC-MS methods such as SIM in conjunction with poly-deuterated tracers or tracers highly enriched in ^{13}C . This may be because GC-MS methods cannot easily be adapted to suit the particular needs of GC-C-IRMS. As mentioned above, the nature of the derivatization agent can influence the accuracy and precision of CSIA, because it will dilute ^{13}C enrichment and can adversely affect chromatographic separation and the efficiency of the combustion catalyst (cf. Table 2) [5**,7,9**].

Seemingly in response to these difficulties, most groups using GC-C-IRMS to study turnover and synthesis rates of various proteins have adopted a strategy whereby a target amino acid is identified that will act as a marker providing unambiguous and quantitative information about the system under investigation. This strategy included the development of a sample preparation protocol tailor-made for the particular amino acid allowing quick and reliable CSIA.

In this way, L-[^{13}C -1]valine was employed to measure in-vivo secretion rates of very low density lipoprotein-

Table 2. Overview of typical analytical procedures used in metabolic and nutritional research involving stable isotopes and their caveats

Compound class	Derivatization	Typical GC conditions ^a	Caveats
Long-chain alcohols and sterols (e.g. cholesterol)	TMS	CP-Sil 5: 50°C (2 min) to 200°C at 40°/min, 200–320°C at 3°/min	None
Fatty acids	Methyl ester using metanol/ BF ₃	CP-Sil 8/CP-Sil 19: 120°C (4 min) to 280°C at 4°/min	None
Hydroxy/amino/keto-carboxylic acids	TMS or tBDMS	CP-Sil 8: 80°C (5 min) to 150°C at 2°/min, 150–220°C at 3.5°/min; 20°/min to 300°C	TMS: multiple derivatives for same compound ^b tBDMS: excessive carbon load ^c
Hydroxy/amino.keto-carboxylic acids	Ethylchloroformates	CP-Sil 24: 60°C (3 min) to 300°C at 6°/min	Risk of non-quantitative derivatization ^d
Hydroxy/amino/keto-carboxylic acids	Acetyl, methylates	CP-Sil 19: 70°C (5 min) to 220°C at 5°/min	None
Amino acids	TMS or tBDMS	CP-Sil 8: 50°C (5 min) to 150°C at 6°/min, 150–300°C at 12°/min	TMS: multiple derivatives for same compound ^b tBDMS: excessive carbon load ^c
Amino acids	TFA, methylates TFA, iso-propylates	CP-Sil 19: 70°C (5 min) to 180°C at 3.5°/min, 180–230°C at 5°/min; 10°/min to 270°C	Poisoning of combustion catalyst ^e
Amino acids	Ethylchloroformates, methylchloroformates	CP-Sil 19: 60°C (3 min) to 100°C at 5°/min, 100–300°C at 10°/min	Risk of non-quantitative derivatization ^d
Amino acids	N-Pivaloyl, iso-propylates	CP-Sil 8: 70°C (1 min) to 200°C at 3°/min, 220–300°C at 10°/min	None
Amino acids	N-Acetyl, propylates	CP-Sil 19: 70°C (5 min) to 200°C at 4°/min, 200–300°C at 6°/min	None

^aThe content of this column should be read as generalized guidelines providing a starting point for the reader to resolve individual analytical tasks. Information provided is choice of gas chromatography (GC) column (=stationary phase) and temperature programme. CP-Sil is a proprietary name of CHROMPACK International BV (Middelburg, the Netherlands) and has been chosen as an example because it is widely recognized.

^bAmbiguity of results; a problem for gas chromatography–mass spectrometry (GC–MS) as well as gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS).

^cStrong dilution of ¹³C enrichment; this affects only GC–C–IRMS measurements. It is, however, only a problem when the measurement cannot be compared against the background or baseline $\delta^{13}\text{C}$ value of an authentic sample.

^dPotential loss of information (GC–MS and GC–C–IRMS).

^eA serious problem for GC–C–IRMS; leads ultimately to incomplete combustion and can negatively affect the accuracy and precision of ¹³C and ¹⁵N isotope abundance measurements.

TFA, Trifluoroacetyl; TMS, Trimethylsilyl; tBDMS, tert-Butyldimethylsilyl.

apolipoprotein B100 in humans [37*], synthesis rates of α_2 -macroglobulin in nephrotic patients [38*] and the protein fractional synthetic rate in skeletal muscle [39*] and plasma albumin [40*].

In the same way, other groups used L-[¹³C-1]leucine to measure the fractional synthesis rate of mixed muscle protein, myosin heavy chain and actin in human skeletal muscle [41*,42], the metabolism of human apolipoprotein B [43] and protein synthesis in patients suffering from rectal cancer [44,45].

To measure collagen synthesis in adult humans, Rennie *et al.* [3] used L-[¹³C-1]proline with an enrichment of 30 mol% excess as the marker amino acid. The usefulness of proline as a tracer lies in the phenomenon of post-translational hydroxylation of collagen-bound proline into 4-hydroxyproline and the fact that all protein-bound 4-hydroxyproline is in collagen.

Menand *et al.* [46] described a method to determine low enrichment levels of free L-[¹³C-1]glutamine in plasma, and Meier-Augenstein *et al.* [12] reported a method to measure [¹³C-1]-labelled BCAA L-valine, L-leucine and L-isoleucine, as well as their transamination products in

human plasma after oral bolus administration. The authors demonstrated that this method could be used to measure whole-body BCAA oxidation in patients suffering from maple syrup urinary disease [12].

High-precision compound-specific isotope analysis of ¹⁵N-labelled amino acids

Published work on studies of amino acid and protein turnover using ¹⁵N isotopic abundance measurements by GC–C–IRMS are still few and far between. This is quite probably a reflection of the analytical challenges associated with high precision CSIA of ¹⁵N isotopic abundance in amino acids. First, the low concentration of nitrogen in amino acids, for amino acids contain two to 11 times more carbon than nitrogen. Second, $\delta^{15}\text{N}$ measurements require N₂, which at least doubles the sample size requirement because two molequivalents of amino acids are needed for the production of one molequivalent of N₂. Non-quantitative sample conversion and reduced sensitivity of the IRMS ion source for N₂ (compared with CO₂) increase sample size requirements further, by a factor of five compared with CSIA of ¹³C-labelled compounds. Finally, even small amounts of atmospheric gas leaks into the GC–C–IRMS instrument result in a high N₂ background level.

However, CSIA of nitrogenous compounds such as amino acids yields new and unexpected insights into the metabolism of organic nitrogen. For example, a study of plasma albumin synthesis using L-[^{13}C -1, ^{15}N]alanine and L-[^{13}C -1, ^{15}N]leucine showed that both amino acids underwent transamination (and re-amination with nitrogen from the body's nitrogen pool) before their incorporation into plasma protein [3]. The observed enrichment ratios of $^{15}\text{N}/^{13}\text{C}$ found in plasma albumin were 1:3.18 and 1:2.29 for alanine and leucine, respectively. A cross-over of ^{13}C could be ruled out because $\delta^{13}\text{C}$ -values for simultaneously administered L-[^{13}C -1]phenylalanine remained at baseline level throughout the course of the study.

Preston *et al.* [47] measured protein synthesis using [^{15}N]glycine to study the effect of ibuprofen on the plasma concentration of acute-phase proteins in patients with colonic cancer. The authors found that ibuprofen caused a significant reduction of all five acute-phase proteins (fibrinogen, C-reactive protein, caeruloplasmin, α_1 -antitrypsin and α_1 -acid glycoprotein) after 3 days of oral ibuprofen.

Monitoring $\delta^{15}\text{N}$ -values of free plasma amino acids from fasting human subjects, Metges and Petzke [48] made the intriguing observation of consistently low levels of ^{15}N abundance ($\delta^{15}\text{N} < 0.0\text{‰}$ versus air) in the amino acids threonine and phenylalanine. The $\delta^{15}\text{N}$ values of all the other amino acids were positive ($\delta^{15}\text{N} > 0.0$ versus air). In subsequent studies that involved the administration of oral bolus doses of [$^{15}\text{N}_2$]urea, Petzke *et al.* [49] found that incorporation of ^{15}N from this source into plasma protein amino acids was consistently low for lysine, histidine, proline and phenylalanine. On the basis of the results of a widened study combining the administration of [$^{15}\text{N}_2$]urea with the ingestion of lactic acid bacteria, Petzke *et al.* [50*] hypothesized that these low levels of ^{15}N incorporation suggest only limited or no participation in the transamination reactions of those amino acids.

Conclusion

High-precision CSIA by GC-C-IRMS of organic compounds at natural abundance and low enrichment levels is a powerful tool that provides new insights as well as quantitative information on human metabolism. GC-C-IRMS is a unique tool that can provide answers to biochemical and physiological questions that cannot be obtained by any other analytical instrumentation.

Acknowledgements

The author gratefully acknowledges financial support through the Dietmar-Hopp Foundation for Medical Research and Studies (Walldorf, Germany). Many thanks are due to Dr Helen F. Kemp (University of Dundee) and Professor J. Thomas Brenna (Cornell University) for critical reading of the manuscript.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

- 1 Koletzko B, Sauerwald T, Demmelmair H. Safety of stable isotope use. *Eur J Pediatr* 1997; 156:S12-S17.
- 2 Preston T, Slater C. Mass-spectrometric analysis of stable-isotope-labeled amino-acid tracers. *Proc Nutr Soc* 1994; 53:363-372.
- 3 Rennie MJ, Meier-Augenstein W, Watt PW, Patel A, Begley IS, Scrimgeour CM. Use of continuous-flow combustion ms in studies of human metabolism. *Biochem Soc Trans* 1996; 24:927-932.
- 4 Brenna JT, Corso TN, Tobias HJ, Caimi RJ. High-precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrom Rev* 1997; 16:227-258.
- One of the best general review articles on IRMS published in recent years. Includes a section dedicated to aspects important for tracer studies such as mass balance, pool mixing and dilution space.
- 5 Meier-Augenstein W. Applied gas chromatography coupled to isotope ratio mass spectrometry. *J Chromatogr A* 1999; 842:351-371.
- This most recent review on GC-C-IRMS gives a comprehensive overview of its wide spectrum of applications. It also provides detailed information on sample preparation, gas chromatographic methods and isotopic calibration.
- 6 Brand WA. High precision isotope ratio monitoring techniques in mass spectrometry. *J Mass Spectrom* 1996; 31:225-235.
- 7 Meier-Augenstein W. The chromatographic side of isotope ratio mass spectrometry-pitfalls and answers. *LC/GC-Mag Sep Sci* 1997; 15:244-253.
- 8 Ellis L, Fincannon AL. Analytical improvements in irm-GC/MS analyses: advanced techniques in tube furnace design and sample preparation. *Org Geochem* 1998; 29:1101-1117.
- 9 Metges CC, Petzke KJ. The use of GC-C-IRMS for the analysis of stable isotope enrichment in nitrogenous compounds. In: *Methods of investigation of amino acid and protein metabolism*, chapter 7. El-Khoury AE (editor). Boca Raton, Florida: CRC Press LLC; 1999; pp. 121-134.
- The only review dedicated to aspects of stable isotope analysis of nitrogenous compounds.
- 10 Koletzko B, Demmelmair H, Hartl W, Kindermann A, Koletzko S, Sauerwald T, Sztanyi P. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. *Early Hum Dev* 1998; 53:S77-S97.
- A very useful reference article, especially regarding questions of safety and dosage of stable isotope tracers used in nutritional and metabolic research.
- 11 Pont F, Duvillard L, Maugeais C, Athias A, Persegol L, Gambert P, Verges B. Isotope ratio mass spectrometry, compared with conventional mass spectrometry in kinetic studies at low and high enrichment levels: application to lipoprotein kinetics. *Anal Biochem* 1997; 248:277-287.
- 12 Meier-Augenstein W, Rating D, Hoffmann GF, Wendel U, Matthiesen U, Schadewaldt P. Determination of ^{13}C enrichment by conventional GC-MS and GC-(MS)-C-IRMS. *Isotopes Environ Health Stud* 1995; 31:261-266.
- 13 Parker RS, Brenna JT, Swanson JE, Goodman KJ, Marmor B. Assessing metabolism of beta-[^{13}C]carotene using high-precision isotope ratio mass spectrometry. *Methods Enzymol* 1997; 282:130-140.
- 14 Rambal C, Pachiaudi C, Normand S, Riou JP, Louisot P, Martin A. Differential incorporation of c-13 label from dietary glucose into neutral sugars of rat intestine macromolecules. *Ann Nutr Metab* 1995; 39:143-151.
- 15 Kloppenburg WD, Wolthers BG, Stellaard F, Elzinga H, Tepper T, deJong PE, Huisman RM. Determination of urea kinetics by isotope dilution with [^{13}C]urea and gas chromatography isotope ratio mass spectrometry (GC-IRMS) analysis. *Clin Sci* 1997; 93:73-80.
- 16 Bunt JEH, Zimmermann LJ, Wattimena JLD, vanBeek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. *Am J Respir Crit Care Med* 1998; 157:810-814.
- An in-vivo study of surfactant phosphatidylcholine palmitate in which [^{13}C]glucose was used as a precursor, infused on the first day of life of preterm infants.
- 17 Rhee SK, Reed RG, Brenna JT. Fatty acid carbon isotope ratios in humans on controlled diets. *Lipids* 1997; 32:1257-1263.
- 18 Brenna JT. Use of stable isotopes to study fatty acid and lipoprotein metabolism in man. *Prostaglandins Leukotrienes Essent Fatty Acids* 1997; 57:467-472.
- 19 Demmelmair H, Vonschenck U, Behrendt E, Sauerwald T, Koletzko B. Estimation of arachidonic-acid synthesis in full-term neonates using natural variation of c-13 content. *J Pediatr Gastroenterol Nutr* 1995; 21:31-36.

6 Assessment of nutritional status and analytical methods

- 20 Carnielli VP, Wattimena DJL, Luijendijk IHT, Boerlage A, Degenhart HJ, Sauer PJJ. The very-low-birth-weight premature-infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids. *Pediatr Res* 1996; 40:169-174.
- 21 Carnielli VP, Sulkers EJ, Moretti C, Wattimena JLD, Vangoudoever JB, Degenhart HJ, et al. Conversion of octanoic-acid into long-chain saturated fatty-acids in premature-infants fed a formula containing medium-chain triglycerides. *Metabolism* 1994; 43:1287-1292.
- 22 Sheaff Greiner RC, Winter J, Nathanielsz PW, Brenna JT. Brain docosahexaenoate accretion in fetal baboons: bioequivalence of dietary alpha-linolenic and docosahexaenoic acids. *Pediatr Res* 1997; 42:826-834.
- 23 Su HM, Bernardo L, Mirmiran M, Ma XH, Corso TN, Nathanielsz PW, Brenna JT. Bioequivalence of dietary alpha-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons. *Pediatr Res* 1999; 45:87-93.
- The latest results in a series of studies that investigated the role of dietary α -linolenic and docosahexaenoic acids as substrates for brain and retinal n-3 fatty acid accretion.
- 24 Binnert C, Laville M, Pachiaudi C, Rigalleau V, Beylot M. Use of gas-chromatography isotope ratio-mass spectrometry to study triglyceride-metabolism in humans. *Lipids* 1995; 30:869-873.
- 25 Stolinski M, Murphy JL, Jones AE, Jackson AA, Wootton SA. Stable-isotope method for determining the gastrointestinal handling of [1-C-13]palmitic acid. *Lipids* 1997; 32:337-340.
- 26 Guo ZK, Nielsen S, Burguera B, Jensen MD. Free fatty acid turnover measured using ultra low doses of [U-C-13]palmitate. *J Lipid Res* 1997; 38:1888-1895.
- 27 Guo ZK, Jensen MD. Intramuscular fatty acid metabolism evaluated with stable isotopic tracers. *J Appl Physiol* 1998; 84:1674-1679.
- A pilot study that used [U-¹³C]palmitate and [¹⁸C-1]oleate to measure intramuscular metabolism of non-esterified fatty acids and triglycerides.
- 28 Croset M, Brossard N, Pachiaudi C, Normand S, Lecerf J, Chirouze V, et al. In-vivo compartmental metabolism of c-13 docosahexaenoic acid, studied by gas-chromatography combustion isotope ratio mass-spectrometry. *Lipids* 1996; 31:S109-S115.
- 29 Demmelmair H, Sauerwald T, Koletzko B, Richter T. New insights into lipid and fatty acid metabolism via stable isotopes. *Eur J Pediatr* 1997; 156:S70-S74.
- 30 Brossard N, Croset M, Normand S, Pousin J, Lecerf J, Laville M, et al. Human plasma albumin transports [C-13]docosahexaenoic acid in two lipid forms to blood cells. *J Lipid Res* 1997; 38:1571-1582.
- 31 Demmelmair H, Baumheuer M, Koletzko B, Dokoupil K, Kratl G. Metabolism of [U-C-13]-labeled linoleic acid in lactating women. *J Lipid Res* 1998; 39:1389-1396.
- By administering [U-¹³C]linolenic acid to lactating women, the origin of long-chain polyunsaturated fatty acids in breast milk was partly identified. Approximately 30% of milk linolenic acid is derived from the diet by direct transfer.
- 32 Cunnane SC, Moine G, Likhodii SS, Vogt J, Corso TN, Brenna JT, et al. [3-C-13] gamma-linolenic acid: a new probe for C-13 nuclear magnetic resonance studies of arachidonic acid synthesis in the suckling rat. *Lipids* 1997; 32:211-217.
- 33 Rhee SK, Kayani AJ, Brenna JT. Desaturation and interconversion of dietary stearic and palmitic acids in human plasma and lipoproteins. *Am J Clin Nutr* 1997; 65:451-458.
- 34 Su HM, Brenna JT. Simultaneous measurement of desaturase activities using stable isotope tracers or a nontracer method. *Anal Biochem* 1998; 261:43-50.
- Microsomal Δ^5 - and Δ^6 -desaturase activities were quantified by measuring the increase in product concentration. The pH and temperature dependency of these reactions were also monitored.
- 35 Sheaff RC, Su HM, Keswick LA, Brenna JT. Conversion of alpha-linolenate to docosahexaenoate is not depressed by high dietary levels of linoleate in young rats-tracer evidence using high-precision mass-spectrometry. *J Lipid Res* 1998; 39:998-1008.
- 36 Menard CR, Goodman KJ, Corso TN, Brenna JT, Cunnane SC. Recycling of carbon into lipids synthesized de novo is a quantitatively important pathway of alpha-[U-C-13]linolenate utilization in the developing rat brain. *J Neurochem* 1998; 71:2151-2158.
- This study showed that, because of a high rate of β -oxidation and carbon recycling, α -linolenate cannot be depended upon as sole source of docosahexaenoate for the developing brain.
- 37 deSainvanderVelden MGM, Rabelink TJ, Gadellaa MM, Elzinga H, Reijngoud DJ, Kuipers F, Stellaard F. In vivo determination of very-low-density lipoprotein-apolipoprotein B100 secretion rates in humans with a low dose of L-[1-C-13]valine and isotope ratio mass spectrometry. *Anal Biochem* 1998; 265:308-312.
- Fractional secretion rate of very low-density lipoprotein-apolipoprotein B100 was measured using a minimal infusion of L-[¹³C-1]valine in conjunction with GC-C-IRMS. The obtained secretion rate of 0.002 ± 0.09 pools/h was in good agreement with that obtained by traditional GC-MS methods (0.23 ± 0.07 pools/h).
- 38 deSainvanderVelden MGM, Rabelink TJ, Reijngoud DJ, Gadellaa MM, Voorbij H, Stellaard F, Kaysen GA. Plasma alpha(2) macroglobulin is increased in nephrotic patients as a result of increased synthesis alone. *Kidney Int* 1998; 54:530-535.
- Combining immunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis with low dosage stable isotope techniques, this study demonstrated that the increased plasma α_2 macroglobulin concentration in nephrotic patients is a result of increased synthesis alone.
- 39 Reijngoud DJ, Hellstern G, Elzinga H, deSainvanderVelden MGM, Okken A, Stellaard F. Determination of low isotopic enrichment of L-[1-C-13]valine by gas chromatography combustion isotope ratio mass spectrometry: a robust method for measuring protein fractional synthetic rates in vivo. *J Mass Spectrom* 1998; 33:621-626.
- A comprehensive GC-C-IRMS method is described to measure protein fractional synthesis rates using L-[¹³C-1]valine as the tracer. The tracer detection limit of this method was 0.0002 (tracer/tracee ratio in mol/mol).
- 40 Kulik W, Meesterburrie JAN, Jakobs C, deMeer K. Determination of delta C-13 values of valine in protein hydrolysate by gas chromatography combustion isotope ratio mass spectrometry. *J Chromatogr B* 1998; 710:37-47.
- A methodological study that demonstrated high linearity of $\Delta^{13}C$ measurements over a wide dynamic range of enrichment (0-1.5 mol% excess).
- 41 Hasten DL, Morris GS, Ramanadham S, Yarasheski KE. Isolation of human skeletal muscle myosin heavy chain and actin for measurement of fractional synthesis rates. *Am J Physiol Endocrinol Metab* 1998; 38:E1092-E1099.
- A simple yet more refined approach was reported for in-vivo studies of muscle contractile protein metabolism. This approach permitted not only the fractional synthesis rate for mixed muscle protein but also myosin heavy chain and actin from small (60-80 mg) human skeletal muscle samples.
- 42 Balagopal P, Ford GC, Ebenstein DB, Nadeau DA, Nair KS. Mass spectrometric methods for determination of [C-13] leucine enrichment in human muscle protein. *Anal Biochem* 1996; 239:77-85.
- 43 Demant T, Packard CJ, Demmelmair H, Stewart P, Bedynek A, Bedford D, et al. Sensitive methods to study human apolipoprotein-b metabolism using stable isotope-labeled amino-acids. *Am J Physiol Endocrinol Metab* 1996; 33:E1022-E1036.
- 44 Hartl WH, Demmelmair H, Jauch KW, Schmidt HL, Koletzko B, Schildberg FW. Determination of protein synthesis in human rectal cancer in situ by continuous [1-C-13]leucine infusion. *Am J Physiol Endocrinol Metab* 1997; 35:E796-E802.
- 45 Hartl WH, Demmelmair H, Jauch KW, Koletzko B, Schildberg FW. Effect of glucagon on protein synthesis in human rectal cancer in situ. *Ann Surg* 1998; 227:390-397.
- 46 Menand C, Pouteau E, Marchini S, Maugere P, Krempf M, Darmaun D. Determination of low C-13-glutamine enrichments using gas chromatography combustion-isotope ratio mass spectrometry. *J Mass Spectrom* 1997; 32:1094-1099.
- 47 Preston T, Fearon KCH, McMillan DC, Winstanley FP, Slater C, Shenkin A, Carter DC. Effect of ibuprofen on the acute-phase response and protein-metabolism in patients with cancer and weight-loss. *Br J Surg* 1995; 82:229-234.
- 48 Metges CC, Petzke KJ. Measurement of N-15/N-14 isotopic composition in individual plasma free amino acids of human adults at natural abundance by gas chromatography combustion isotope ratio mass spectrometry. *Anal Biochem* 1997; 247:158-164.
- 49 Petzke KJ, Korkushko OV, Semesko TM, Metges CC. N-isotopic composition in human plasma protein amino acids at natural abundance level and after a single [N-15(2)]urea administration measured by GC-C-IRMS. *Isotopes Environ Health Stud* 1997; 33:267-275.
- 50 Petzke KJ, Grigorov JG, Korkushko OV, Kovalenko NK, Semesko TG, Metges CC. Incorporation of urea nitrogen into fecal protein and plasma protein amino acids in elderly human volunteers after ingestion of lactic acid bacteria. *Z Ernahrungswiss* 1998; 37:368-375.
- The beneficial health effects of fermented milk products may be linked to the incorporation of urea nitrogen into plasma protein amino acids as a result of the metabolic activity of ingested lactic acid bacteria.

Department of Communications, Information Technology and the Arts

Anti-Doping Research Program

Progress Report

March 2004

**Project: Statistical Population Studies to Support New Analytical
Methodologies using the EPO2000 Project Urine Samples.**

Investigators

R. Kazlauskas, G. J. Trout, C. Howe and J. Rogerson (Australian Sports Drug
Testing Laboratory)